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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Signaling by the four members of the erbB family of receptor tyrosine kinases involves their ligand-induced homo- and/or hetero-oligomerization. Ligand-induced receptor homodimerization appears to be driven straightforwardly by receptor extracellular domains, and can be recapitulated *in vitro*. By contrast, hetero-oligomerization cannot be detected in studies of isolated extracellular domains, and has only been observed for receptors in cellular membranes. We were therefore interested in determining which domains of an erbB receptor drive hetero-oligomerization. By analyzing a series of breast cancer cell-lines we found that EGF does not induce robust phosphatidylinositol-3-kinase (PI-3-K) activation in MCF-7 cells, since these cells express little to no EGF receptor. We visualize PI-3-K activation *in vivo* by observing the cytoplasm-to-plasma membrane translocation of a pleckstrin homology domain (fused to green fluorescent protein) that specifically recognized $PtdIns(3,4,5)P_3$ (Grp1). We found that overexpression of wild-type EGFR in MCF-7 cells 'restored' the ability of EGF to induce robust PI-3-K activation in these cells. Surprisingly, both a kinase-deficient EGFR mutant and a form of EGFR lacking all cytoplasmic sequences were equally effective in mediating EGF-induced PI-3-K activation. The EGFR extracellular domain 'tethered' to the plasma membrane by a GPI anchor, or just added in excess (together with EGF) were also capable of inducing PI-3-K activation.

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INTRODUCTION

Our long-term goal is to understand the mechanism of signal transduction initiated upon ligand binding to the erbB family of receptor tyrosine kinases (RTKs), which includes the epidermal growth factor (EGF) receptor (erbB1 or EGFR), the Neu oncogene product (erbB2 or HER2) as well as erbB3 and erbB4. *In vitro* studies of the isolated extracellular domains of these receptors have begun to shed light on how these receptors form homooligomers in the presence of their cognate growth factor ligands. However, there is still no real understanding of how heterooligomers form. The combination of RTK expression and the presence of growth factors determine a cell's fate, directing it towards such diverse behaviors as proliferation, differentiation, motility, or even apoptosis. As heterooligomerization of the erbB RTK family has been strongly implicated in oncogenesis, this is an important process to understand. We have developed a fluorescence microscopy-based method to monitor phoshatidylinositol-3-kinase (PI3K) activation in living cells. We first used this method to examine the effects of different erbB receptor ligands on cells expressing a range of complements of erbB RTKs. We are currently using this technique to illuminate which portions of the erbB receptors are necessary for ligand-induced activation of downstream signaling cascades.

BODY

Aim 1 of the original Statement of Work was to "determine ligand-specificity of PI3K activation *in vivo*. The first step involved in this first Aim was to examine the effects of epidermal growth factor (EGF) and neuregulin-1β1 (NRG) on various cell-lines expressing different endogenous levels of the erbB receptor tyrosine kinases (RTKs). We tested five commonly used breast-cancer cell-lines with our fluorescence microscopy-based PI3K activation assay, in which we monitor the cytoplasm-to-plasma membrane translocation of an enhanced green fluorescent protein (EGFP)-Grp1 pleckstrin homology (PH) domain fusion protein. The results from these five cell-lines are appended as Figure 1. The second step of Aim 1 was to perform co-immunoprecipitations in these cell-lines to examine the effects of EGF and NRG on the formation of erbB receptor homo- and heterooligomers as well as complexes between the erbB receptors and phosphatidylinositol-3-kinase (PI3K). We found that there were significant cell-line differences in the complements of complexes formed, but could draw no significant conclusions from these results.

Aim 2 of the Statement of Work was to "identify the necessary components of receptor oligomerization." In essence, the steps outlined in this Aim were to utilize the information obtained from the experiments in Aim 1 in order to generate meaningful reagents, including stable cell-lines expressing different erbB receptors or receptor chimerae, in order to identify which components of the receptors were required for oligomerization. As can be seen in Figure 1, both EGF and NRG activated PI3K in all five cell-lines tested, except the MCF-7 cell-line, which lacked a response to EGF. We hypothesized that these cells did not respond to EGF due to their extremely low expression level of erbB1, the EGF receptor. We therefore generated a derivative of the parental MCF-7 cell-line stably expressing erbB1, which demonstrated a restored PI3K response to EGF, as assayed by the EGFP-Grp1 PH translocation assay. Based on this result, we felt that the MCF-7 cell-line provided an ideal system for determining which domain(s) of erbB1 were required for transducing an EGF signal. We therefore made a number of derivative stable cell-lines, overexpressing various forms of erbB1 including "K271M," a kinase-dead mutant (a mutant of the full-length receptor in which a critical lysine in the kinase domain has been mutated to a methionine), "erbB1myc," a truncated form of the receptor in which the entire cytoplasmic domain has been replaced by the c-myc peptide tag, and "GPI," another truncation of the receptor in which the entire receptor C-terminal to the extracellular domain has been replaced with a glycophosphoinositol lipid linkage. All three of these stable cell-lines demonstrated PI3K activation in response to EGF, as is shown in Figure 2. Given the demonstration that simply expressing the extracellular domain of erbB1 at the cell surface was sufficient to restore a PI3K response in MCF-7 cells, we returned to the parental MCF-7 cells, but we included recombinant soluble erbB1 extracellular domain (s-erbB1) as part of the stimulant mixture. As can be seen in Figure 3, we were able to demonstrate a dose-dependent response to s-erbB1, but only when EGF was also present.

Finally, there are a number of ligands which bind specifically to erbB1. These include EGF itself, transforming growth factor-alpha (TGFα), and amphiregulin (AR), as well as ligands which bind and activate both erbB1 and erbB4, including betacellulin (BTC) and heparin-binding EGF-like growth factor (HB-EGF). We tested the ability of all of these ligands to activate PI3K in the MCF-7 parental and stable cell-lines with the EGFP-Grp1 PH translocation assay. As we expected, both BTC and HB-EGF activated PI3K in the parental, as well as the stable cell-lines, since the MCF-7 cells express erbB4. However, we did not see a response to AR or TGFα in the parental MCF-7 cells. TGFα was capable of stimulating PI3K activity in all of the stable cell-lines, similarly to EGF, whereas AR was only able to activate PI3K in the stable cell-lines expressing full-length erbB1 isoforms (wild-type erbB1 and K271M). These

results are summarized in Figure 4. This data is consistent with reports in the literature that AR requires residues in the C-terminal tail, but not functional kinase activity, of erbB1, for effective signal transduction.

After collecting all the above data, we were interested in obtaining biochemical verification of the PI3K activation, rather than simply relying on the EGFP-Grp1 PH translocation assay. There are a number of downstream effector molecules that become phosphorylated after PI3K is activated, including Akt/PKB and $p70^{S6K}$. We therefore stimulated the MCF-7 cell-lines (parental and stable) with EGF and examined the phosphorylation state of these drownstream effector molecules in the lysates. We were never able to conclusively demonstrate a difference in the activation of these effectors between the different cell-lines. Given that we could not obtain solid biochemical data, we next attempted double-blind EGFP-Grp1 PH translocation studies. At this point, we began to note similar, robust levels of PI3K activation in both the parental and stable cell-lines when stimulated with EGF. We do not have a conclusive explanation at this time as to why we seem to have regained a response to EGF in the parental cell-lines. We currently feel that the results of the EGFP-Grp1 PH translocation assays were artifacts due to operator error, or that we had originally isolated a sub-clone of the MCF-7 cell-line that was particularly resistant to EGF stimulation since the MCF-7 cell-line is known to mutate quite rapidly and dramatically. At the current time we are examining different, more robust cell-lines to determine if we can identify a similar response to EGF + s-erbB1.

Aim 3 of the Statement of Work was to "monitor ligand-induced receptor co-patching on the cell surface." The steps involved in this aim were to generate fusions of the erbB receptors with variants of EGFP in order to monitor fluorescence co-patching and/or fluorescence resonance energy transfer (FRET) between the receptor fusions in living cells. We have generated constructs to express both full-length and truncation mutants of all four erbB receptors fused to EGFP as well as both ECFP (cyan) and EYFP (yellow), the commonly used FRET fluorophore pair. We have as of yet not performed co-patching experiments and have not been able to monitor FRET in living cells, even using positive controls obtained from other labs.

KEY RESEARCH ACCOMPLISHMENTS

- Examined PI3K activation by EGF and NRG in five breast cancer cell-lines
- Identified MCF-7 cell-line as model system for determining which domains of erbB1 are required for transducing an EGF signal
- Generated stable derivatives of MCF-7 cell-line expressing various forms of erbB1, including wild-type, kinase deficient, cytoplasmic domain truncation, and extracellular domain fused to outer leaflet of plasma membrane via GPI-linkage
- Tested all stable MCF-7 cell-lines for PI3K activation by EGFP-Grp1 PH translocation assay using EGF, TGFα, AR, BTC and HB-EGF – found that BTC and HB-EGF initiate signaling even in parental cells (probably through endogenous erbB4), EGF and TGFα require only presence of erbB1 ectodomain to be expressed, and AR requires expression of the cytoplasmic domains, but not kinase activity, to induce PI3K activity
- Generated fusions of all four erbB receptors, and truncation mutants lacking entire cytoplasmic domains, to ECFP, EGFP and EYFP.

REPORTABLE OUTCOMES

There are no reportable outcomes based on this work.

CONCLUSIONS

The commonly held view in the literature is that all four of the erbB receptors can form homo- and heterodimers in response to binding growth factor. However, our lab has considered a different model of erbB receptor activation. We have shown *in vitro* that the soluble extracellular domains of erbB1 and erbB4 both homooligomerize efficiently upon binding to their cognate ligands, whereas we see neither homooligomerization of erbB2 or erbB3, or heterooligomerization of any of the erbB receptors. These observations, along with a number of pieces of data from the literature, led us to question if the receptors form signaling-competent species in the form of higher-order oligomers, such as heterotetramers. In this paradigm, erbB1 and erbB4 serve as central receptors, capable of forming homodimers in the presence of EGF or NRG, respectively. These homodimers would then surve as "ligands" or condensation points capable of inducing heterooligomers with the other erbB ligands.

We have utilized a novel *in vivo* fluorescence microscopy-based assay to examine differences in EGF- and NRG-stimulation of PI3K activity in different breast cancer cell-lines, as well as in stable derivatives of the MCF-7 breast cancer cell-line. Our results ultimately demonstrate that PI3K can be activated in MCF-7 cells by EGF as long as the erbB1 extracellular domain is present, even if in the form of recombinant soluble protein. These results support the heterotetramer hypothesis. However, we cannot rule out the possibility that the PI3K response that we have seen is due to the activation of an unknown receptor that specifically binds EGF-bound erbB1 extracellular domain, rather than the induction of higher-order oligomers of the erbB RTKs by the s-erbB1.

Soluble erbB receptor extracellular domains are often noted in the sera of human subjects, especially those with advanced stage cancers. Pathologists have noted, for instance, a strong correlation between the presence of soluble erbB2 extracellular domain and breast cancer. It has been hypothesized that cells shed the extracellular domains of their receptors in order to provide a means to "soak up" excess growth factors present around the cells. Our data, however, indicate that these extracellular domains may actually serve as a signaling intermediate. This could therefore provide an interesting and useful area for further research and potential drug targets for novel chemotherapeutic agents.

REFERENCES

No references are provided.

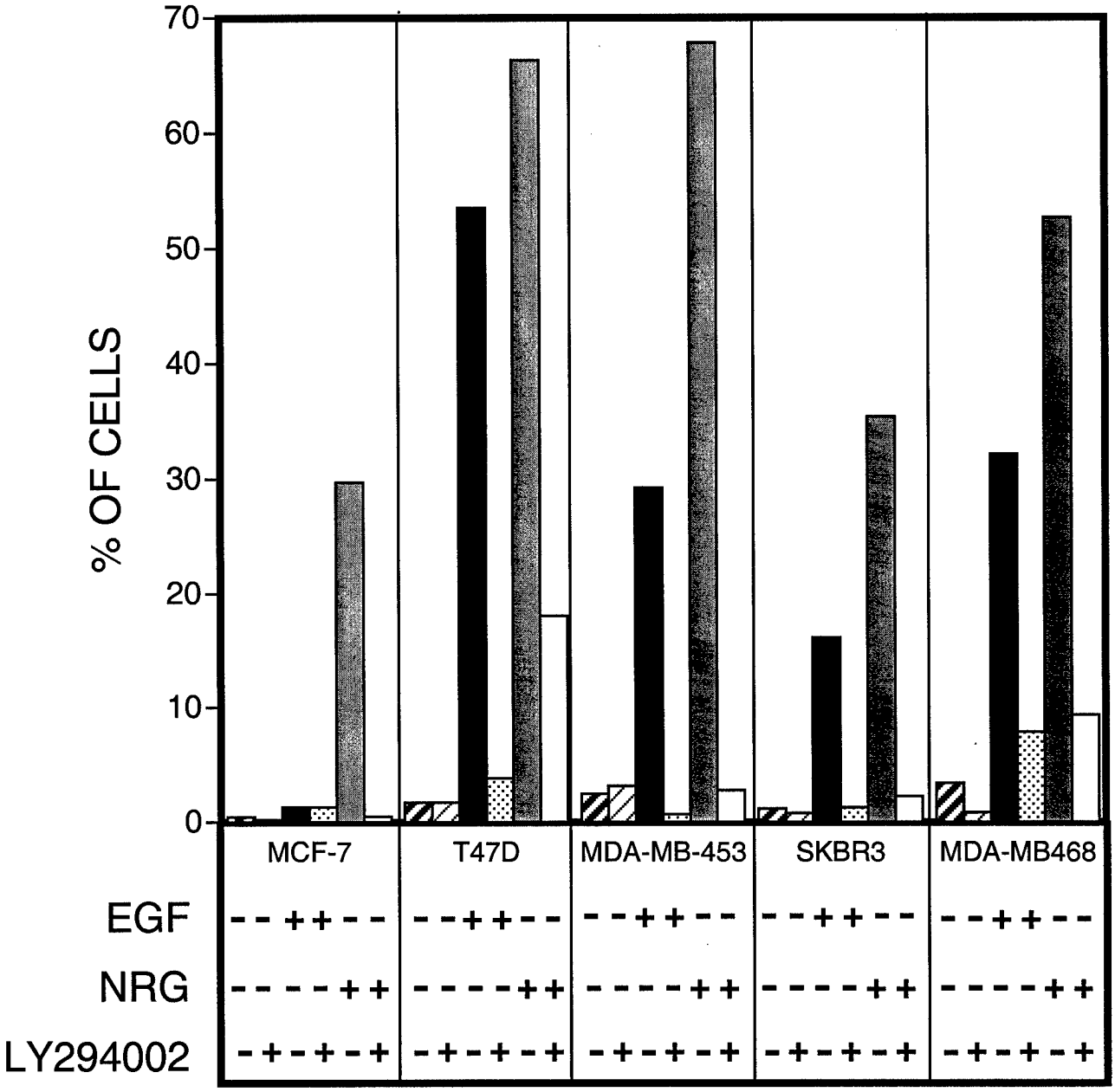

70
60
50
40
30
20
10
0
% OF CELLS
MCF-7
T47D
MDA-MB-453
SKBR3
MDA-MB468
EGF
NRG
LY294002

FIGURE 1

FIGURE 2

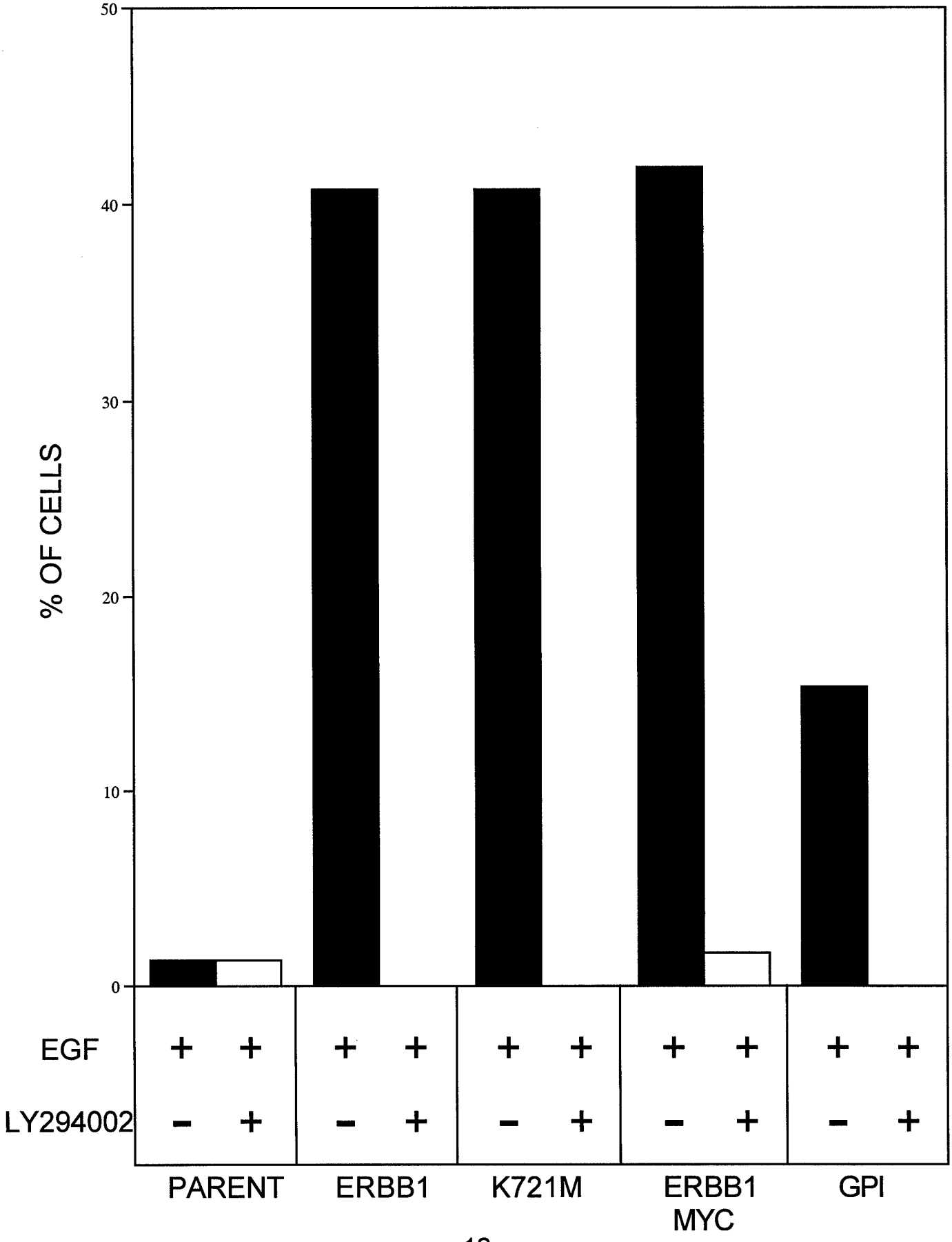
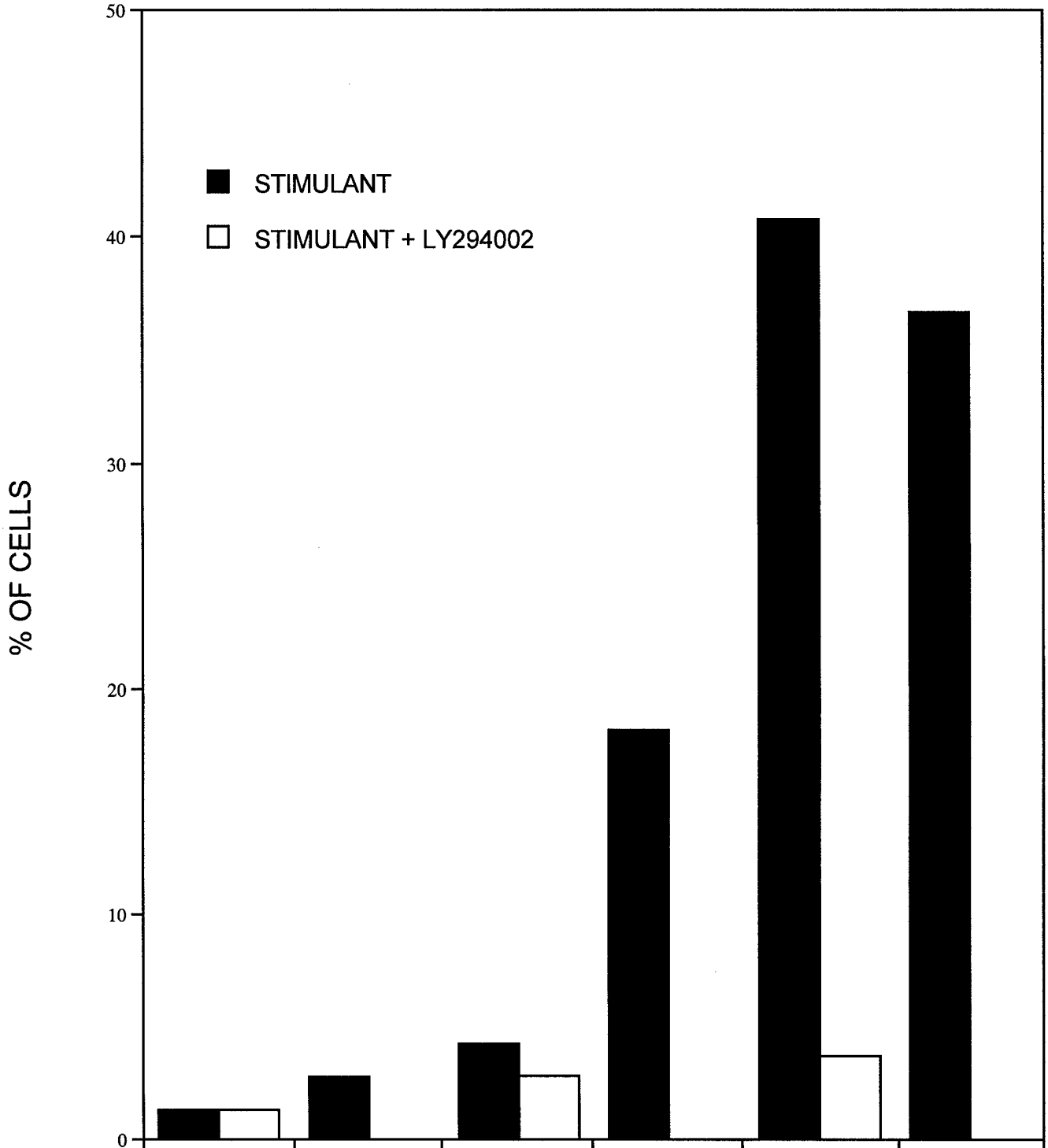
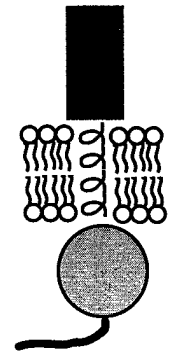

50
40
30
20
10
0
% OF CELLS
EGF
LY294002
+ + + + + + + + + +
- + - + - + - + - +
PARENT
ERBB1
K721M
ERBB1 MYC
GPI

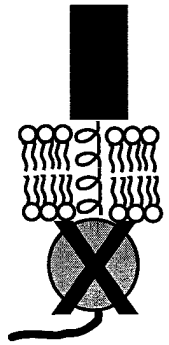
FIGURE 3

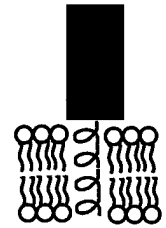


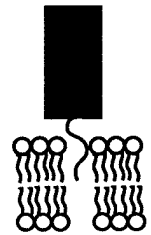
[EGF] (ng/mL)	100	0	100	100	100	100
[s-erbB1] (pM)	0	928	8.99	45	*92.8*	899

FIGURE 4









	EGF/TGFα	AR
erbB1	+	+
erbB1 K721M	+	+
erbB1myc	+	-
GPI-B1ecto	+	Not Tested